

Bioactive Benzopyrone Derivatives from New Recombinant Fusant of Marine *Streptomyces*

Mervat M. A. El-Gendy · M. Shaaban ·
A. M. EL-Bondkly · K. A. Shaaban

Received: 26 August 2007 / Accepted: 21 February 2008 /
Published online: 13 June 2008
© Humana Press 2008

Abstract In our searching program for bioactive secondary metabolites from marine *Streptomyces*, three microbial benzopyrone derivatives (**1–3**), 7-methylcoumarin (**1**) and two flavonoides, rhamnazin (**2**) and cirsimaritin (**3**), were obtained during the working up of the ethyl acetate fraction of a marine *Streptomyces* fusant obtained from protoplast fusion between *Streptomyces* strains Merv 1996 and Merv 7409. The structures of the three compounds (**1–3**) were established by nuclear magnetic resonance, mass, UV spectra, and by comparison with literature data. Marine *Streptomyces* strains were identified based on their phenotypic and chemotypic characteristics as two different bioactive strains of the genus *Streptomyces*. We described here the fermentation, isolation, as well as the biological activity of these bioactive compounds. The isolated compounds (**1–3**) are reported here as microbial products for the first time.

Keywords Marine *Streptomyces* · New recombinant fusant · Benzopyrones · Antibacterial and antifungal agents

M. M. A. El-Gendy (✉)
Department of Chemistry of Natural and Microbial Products, Division of Pharmaceutical Industries,
National Research Centre, El-Beheos st. 33, Dokki-Cairo 12622, Egypt
e-mail: m_morsy_70@yahoo.com

M. Shaaban
Department of Chemistry of Natural Compounds, Division of Pharmaceutical Industries,
National Research Centre, El-Beheos st. 33, Dokki-Cairo 12622, Egypt

A. M. EL-Bondkly
Applied Microbial Genetics Laboratory, Genetics and Cytology Department, National Research Centre,
El-Beheos St. 33, Dokki-Cairo 12622, Egypt

K. A. Shaaban
Department of Organic Chemistry, University of Göttingen, Tammannstrasse 2, 37077 Göttingen,
Germany

Introduction

During the past 30 years, a large number of new compounds with structures completely different from those isolated from terrestrial organisms were successfully discovered from marine sources [1]. The filamentous bacteria, *Streptomyces* species, are well known by a linear chromosome, complex morphological differentiation, and an ability to produce many bioactive secondary metabolites, containing important compounds for pharmaceutical and agrochemical uses [2].

The genetic manipulation via protoplast fusion protocol is considered as a recent method to promote recombination in a variety of prokaryotic and eukaryotic microorganisms. The protoplast fusion technique is an essential method to exchange genetic material and to obtain genetic recombination. It often plays an important role in industrial strain improvement programs and in constructing a recombinant culture characterized by the properties inherited from their parental cultures and also by new properties. This is most likely reflected positively on the productivity and diversity of their secondary metabolites [3–5]. Moreover, the fusion products may be selected by a specifically designed strategy, e.g., nutritional complementation of auxotrophs or detectable resistance to antibacterial agents [6].

Protoplast fusion between different genotype marine *Streptomyces* strains was proved to be an effective tool to construct new recombinants with new characters. Many authors studied the effect of protoplast fusion on antibiotics productivity, and many authors studied *Streptomyces* strains as a tool in gene transfer to construct new recombinants [3–6].

Benzopyrone derivatives are widely distributed in plants (free or as heterosides), and they have multibiological activities. Flavonoids, polyphenolic compounds, are highly antioxidant [7–10], antitumor, and antiatherosclerosis [11–19] substances. On the other hand, coumarins play an important role as anticoagulants, estrogenic, anti-human immunodeficiency virus, antitumor, antihypertension, antiarrhythmia, dermal photosensitizing, antimicrobial, vasodilating, molluscicidal, antihelminthic, sedative and hypnotic, analgesic, antiseptic, and antiosteoporosis substances, which provide pain relief, prevent asthma, and have a hypothermic activity [20, 21]. Recently, they are considered as a prevention factor of hepatocellular carcinomas [22]. Although these types of compounds are commonly present in many dicotyledonous families, including the *Apiaceae*, *Asteraceae*, *Fabiaceae*, *Moraceae*, *Rosaceae*, *Rubiaceae*, *Rutaceae*, and *Solanaceae* [23–25], they are very limited as microbial products, especially from bacteria [26].

The present work was a trial to use the protoplast fusion technique between two different genomes of marine *Streptomyces* strains to construct recombinants with new characters, which have the abilities to produce different unusual microbial products. We reported here the isolation and structure determination of three microbial metabolites, 7-methylcoumarin (**1**), cirsimaritin (**2**), and Quercetin-7,3'-dimethylether (**3**), from the fusant of marine *Streptomyces* strains.

Materials and Methods

Instrumental Analysis

Nuclear magnetic resonance (NMR) spectra were measured on AMX 300 (300.135 MHz), Varian Unity 300 (300.145 MHz), and Varian Inova 600 (150.7 MHz) spectrometers. Electron impact mass spectrometry (EI-MS) spectra were recorded on a Finnigan MAT 95

spectrometer (70 eV) with perfluorokerosine as the reference substance for electron impact high-resolution mass spectrometry (EI HRMS). Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 Series Fourier transform IR spectrometer from KBr pellets. UV-VIS spectra were recorded on a Perkin-Elmer Lambda 15 UV/VIS spectrometer. Flash chromatography was carried out using silica gel (30–60 μm). R_f values were measured on Silica gel TLC (Kieselgel 60 F 254, 0.20 mm, Merck). Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia).

Microorganisms and Cultivation

Strains Merv1996 and Merv7409 were isolated from jellyfish samples collected from the El-Agami coast of the Mediterranean Sea, Egypt. The samples were collected in sterile bottles and deposited in the Department of the Chemistry of Natural and Microbial Products, National Research Centre, Egypt, and kept in refrigerator (5 °C). The marine *Streptomyces* isolates were selected using agar medium (YMG) containing (g/L): yeast extract (4.0), malt extract (2.0), glucose (10.0), agar (20.0), and 75% seawater [2]. Spread plates were incubated at 28 °C for 2 weeks, and the single colonies were reinoculated in the same medium and examined for its purity. Strains Merv1996 and Merv7409 were identified by the phenotypic [27–29] and the chemotypic [30, 31] characteristics.

Formation of Protoplasts and Protoplast Fusion

Two different marine *Streptomyces* strains were tested against five antibacterial agents, and their responses were recorded and used as markers for fusants detection after protoplast fusion. Protoplasts were prepared, fused, and regenerated [6, 32, 33].

Bioautography of the Bioactive Compounds

One milliliter of the well-grown preculture of the marine *Streptomyces* fusants and their parents was inoculated into 100 ml of the YMG liquid medium in a 500-ml flask and cultured at 28 °C for 7 days. Each broth filtrate was extracted with ethyl acetate, dehydrated with Na_2SO_4 , and concentrated to crude extracts. The extracts were applied to thin-layer chromatography (TLC) (Kieselgel 60 F254, Merck) and developed with chloroform–methanol (15:1). The developed TLC plate was dried and contacted with a bioassay plate for 30 min and incubated at 28°C overnight. The bioassay plate was composed of two layers; the bottom layer contained YMG–agar medium containing 1.5% agar, while the top layer contained YMG–agar medium (0.8%) supplemented with 2% of the overnight culture of the indicator microorganisms [2].

Fermentation and Isolation of Bioactive Compounds

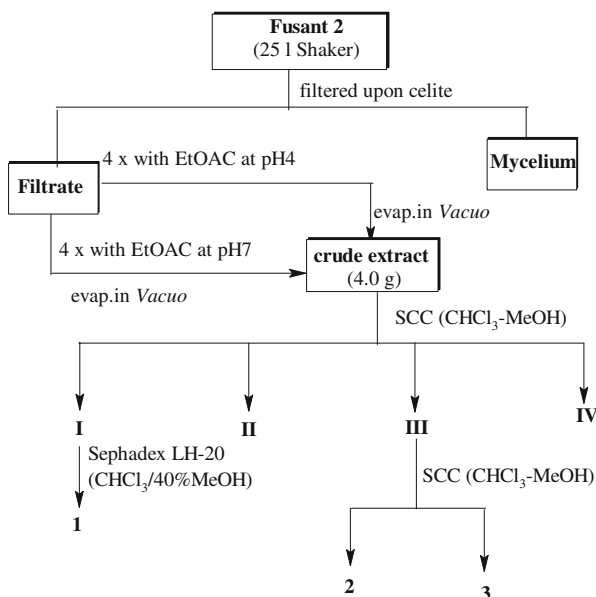
Well-grown agar subcultures of the marine *Streptomyces* fusant (F2) were used to inoculate five 250-ml Erlenmeyer flasks each containing 50 ml of the YMG medium without agar for 7 days. The obtained preculture was used to inoculate 100 1-l Erlenmeyer flasks each containing 250 ml of the same medium using a rotary shaker (200 rpm) for 7 days at 28°C. The resulting faint yellow broth was harvested and mixed with diatomaceous earth (celite, approximately 1 kg) and filtered under a vacuum. The filtrate was extracted with ethyl acetate at pH 4.0 and 7.0, respectively. Both ethyl acetate fractions confirmed as identical after TLC were combined and evaporated to dryness under reduced pressure, yielding a

brownish yellow extract (4.0 g). The extract was chromatographed on flash silica gel column, eluted with a CHCl_3 –MeOH gradient. With the aid of TLC monitoring, four fractions were delivered, I (0.75 g), II (0.31 g), III (1.32 g) and IV (1.52 g). Fraction I was applied to the Sephadex LH-20 column chromatography eluted with CHCl_3 /MeOH (6:4) leading to the isolation of 12 mg of the colorless crystal of compound **1**. Further purification of fraction III by silica gel column chromatography using the CHCl_3 –MeOH gradient afforded two yellow solids of compound **2** (15 mg) and compound **3** (18 mg; Fig. 1).

The characteristics of 7-methylcoumarin (**1**) are as follows: $\text{C}_{10}\text{H}_8\text{O}_2$, bright white needle crystals, Mp 179–181 °C; UV-absorbing (360 nm) substance, showed no color staining with anisaldehyde/sulfuric acid or concentrated sulfuric acid; R_f , 0.90 (Benzne/50% CHCl_3); ^1H NMR (300 MHz, CDCl_3): δ 7.68 (d, $J=9.5$ Hz, 1H, H-4), 7.37 (d, $J=7.8$ Hz, 1H, H-5), 7.17 (d, $J=1.1$ Hz, 1H, H-8), 7.10 (dd, $J=7.8, 1.1$ Hz, 1H, H-6), 6.35 (d, $J=9.5$ Hz, 1H, H-3), 2.45 (s, 3H, 7- CH_3); ^{13}C NMR (75.5 MHz, CDCl_3): δ 161.1 (C_q -2), 154.1 (C_q -8a), 143.4 (CH-4), 143.1 (C_q -7), 127.5 (CH-5), 125.6 (CH-6), 116.4 (C_q -4a), 115.4 (CH-3), 21.7 (7- CH_3); EIMS (70 eV): m/z (%) 160 $[\text{M}]^+$ (100), 145 $[\text{M}-\text{CH}_3]^+$ (2), 132 $[\text{M}-\text{CO}]^+$ (72), 131 $[\text{M}-\text{CHO}]^+$ (44), 104 (18), 103 (12), 77 (12), 63 (8), 51 (8); EI HRMS m/z 160.05187 (calcd for M^+ , $\text{C}_{10}\text{H}_8\text{O}_2$, 160.05188).

The characteristics of rhamnazin; quercetin-7,3'-dimethylether (**2**) are as follows: $\text{C}_{17}\text{H}_{14}\text{O}_7$, yellow powder, Mp 216–218 °C; UV-absorbing (254 nm) and UV-deep yellow fluorescence; R_f , 0.87 (CHCl_3 /5% MeOH); 0.80 (Benzne/50% CHCl_3); UV/vis (MeOH) $\lambda_{\text{max}}=267, 336$; (MeOH/NaOMe): 267, 379, 401; (AlCl_3 /MeOH): 275, 302, 400; (NaOAc/MeOH): 268, 340 nm; ^1H NMR (300 MHz, DMSO- d_6): δ 12.83 (s, 1H, 5-OH), 8.17 (d, $J=1.8$ Hz, 1H, H-2'), 8.14 (d, $J=8$ Hz, 1H, H-6'), 7.12 (d, $J=8$ Hz, 1H, H-5'), 6.92 (s, 1H, H-8) 3.72 (s, 3H, 3'- OCH_3), 3.69 (s, 3H, 7- OCH_3); EIMS (70 eV): m/z (%) 330 $[\text{M}]^+$ (100%), 315 $[\text{M}-\text{CH}_3]^+$ (75%), 300 $[\text{M}-(2\text{CH}_3)]^+$ (28%), 287 (22%), 271 (18%), 259 (34%), 243 (8%), 231 (9%), 215 (12%), 203 (5%), 73 (12%), 60 (8%), 43 (7%).

Fig. 1 Working-up scheme of the fusant *Streptomyces* (F2)



The characteristics of cirsimaritin; 6-methoxyapigenin 7-methylether (**3**) are as follows: $C_{17}H_{14}O_6$, yellow powder, Mp 258–259 °C; UV-absorbing (254 nm) and UV- deep yellowish green fluorescence; R_f , 0.50 ($CHCl_3$ /5% MeOH); 0.36 (Benzene/50% $CHCl_3$); UV/vis (MeOH) λ_{max} =276, 334; (2M NaOH): 280, 367; (5% $AlCl_3$ /MeOH): 275, 302, 361; ($AlCl_3$ /HCl): 268, 301, 353 nm; 1H NMR (300 MHz, DMSO- d_6): δ 12.85 (s, 1H, 5-OH), 7.95 (d, $^2J=8$ Hz, 2H, H-2',6'), 6.95 (d, $^2J=8$ Hz, 2H, H-3',5'), 6.92 (s, 1H, H-8), 6.80 (s, 1H, H-3), 3.95 (s, 3H, 6-OCH₃), 3.85 (s, 3H, 7-OCH₃); EIMS (70 eV): m/z (%): m/z 314 ($[M]^+$, 100%), 299 ($[M-CH_3]^+$, 75%), 271 ($[M-(CO+CH_3)]^+$, 28%), 268 (22%), 181 (18%), 153 (34%), 119 (8%), 73 (9%), 60 (12%).

Evaluation of Biological Activity

Antimicrobial activities of secondary metabolites produced by marine *Streptomyces* and their fusants were examined by the agar diffusion methods using an antibiotic assay of Whatman product no. 2017, 6 mm in diameter. The antimicrobial potentialities were estimated by measuring the diameter of inhibition zones formed on the agar plates of the target strains. They are *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 6538), *Micrococcus luteus* (ATCC 9341), *Rhodotorula acuta*, *Pichia angusta*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus niger*, and *Botrytis fabae*. This was carried out using Luria broth medium containing peptone (10.0 g/l), yeast extract (5.0 g/l), NaCl (10.0 g/l) at 37 °C for bacteria and potato dextrose agar (Merck) for fungi at 28 °C. The minimum inhibition concentration (MIC) of the pure compounds was expressed as microgram per milliliter by the dilution method [27].

Results and Discussion

Taxonomy of the Producing Strains

While electron microscopic examination of strain Merv 1996 revealed nonfragmented branched mycelium with rectiflexibles chains of less than ten smooth nonmotile orthospores, strain Merv 7409 showed hooked chains of more than 20 hairy nonmotile orthospores. Analysis of the whole cell hydrolysates of both strains revealed the presence of the LL isomer of diamino pimelic acid and the sugar ribose only (cell wall of type 1). On the other hand, these strains showed high G+C content (more than 72%). These analytical data indicated that marine Merv 1996 and Merv 7409 strains represent two strains of the genus *Streptomyces* [28–31].

Protoplast Fusion and Evaluation of Fusants Productivity

The fusion was carried out between two different marine *Streptomyces* strains (Merv 1996 × Merv 7409). Biological (agar diffusion test method), chemical (TLC), and bioautography assays of the two strains established their complete difference in their metabolic products. Strain Merv 1996 exhibited antibacterial activity against Gram-positive bacteria, while strain Merv 7409 displayed activity only against yeasts and filamentous fungi. Moreover, in chemical screening using TLC, the Merv 1996 extract displayed an UV absorption middle polar band (bright sky blue) with no coloration by spraying with sulfuric acid reagent and heating, to which the antibacterial activity might be attributed. On the other hand, strain of

Merv 7409 displayed two yellow middle polar bands, which showed no color change with diluted sodium hydroxide, pointing to nonhydroxyquinones.

To investigate the effect of the protoplast fusion technique as a tool to produce new recombinant isolates with new desirable characters, the antibiotic resistance or sensitivity of the two marine *Streptomyces* strains were studied against five antibacterial agents (ampicillin, 100 $\mu\text{g/ml}$; mitomycin, 5 $\mu\text{g/ml}$; vancomycin, 75 $\mu\text{g/ml}$; tetracycline, 30 $\mu\text{g/ml}$ and kanamycin, 50 $\mu\text{g/ml}$). The results displayed that the marine *Streptomyces* Merv 1996 strain is resistant to mitomycin, ampicillin, and tetracycline as well as sensitive to vancomycin and kanamycin. On the other hand, marine *Streptomyces* Merv 7409 strain exhibited resistance to vancomycin and ampicillin as well as sensitivity to mitomycin, kanamycin, and tetracycline. In a previous work [3, 34], different responses of different isolates for one or more antibacterial agents were used as markers in the protoplast fusion. This different antibiotics response of the two marine strains was applied as selective markers during the detection of fusants after protoplast fusion. The two marine *Streptomyces* strains were forced to protoplasting. Figure 2 shows protoplasts and mycelia.

After the fusion procedure, 11 colonies were proved to have the ability to grow on the selective medium containing mitomycin, vancomycin, and tetracycline, confirming the true recombinants of the two marine strains, Merv 1996 and Merv 7409. The results in Table 1 show the inhibition zone diameter (mm) of the two parents and their fusants. It appeared that out of the 11 fusants, only the two recombinants F2 and F9 showed potent bioactivity

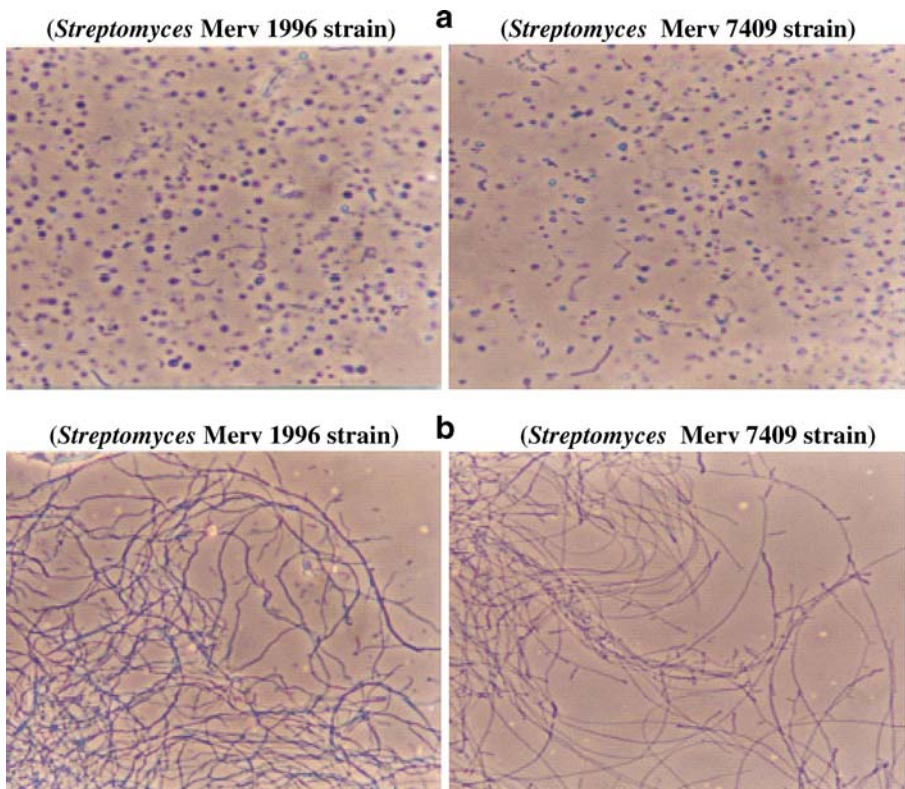


Fig. 2 The formation of marine *Streptomyces* protoplasts (a) in comparison with its mycelia (b)

Table 1 Comparison of antimicrobial spectrum and activities between parental strains and their created fusants.

Parental and fusants	Inhibition zone diameter (mm)											
	Gram-negative bacteria		Gram-positive bacteria					Yeasts			Filamentous fungi	
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>Sta. aureus</i>	<i>M. luteus</i>	<i>R. minuta</i>	<i>P. angusta</i>	<i>C. albicans</i>	<i>C. neoformans</i>	<i>A. niger</i>	<i>B. fabae</i>	
P1	0	0	22	30	25	0	0	0	0	0	0	
P2	0	0	0	0	0	32	28	20	23	28	25	
F1	0	0	22	30	25	32	28	20	23	0	0	
F2	0	0	22	30	25	32	28	20	23	28	25	
F3	0	0	22	30	25	0	0	0	0	28	25	
F4	0	0	22	30	25	32	28	20	23	0	0	
F5	0	0	0	0	0	32	28	20	23	28	25	
F6	0	0	22	30	25	32	28	20	23	0	0	
F7	0	0	22	30	25	0	0	0	0	0	0	
F8	0	0	0	0	0	32	28	20	23	28	25	
F9	0	0	22	30	25	32	28	20	23	28	25	
F10	0	0	0	0	0	32	28	20	23	28	25	
F11	0	0	22	30	25	0	0	0	0	0	0	

P1 Streptomyces Merv 1996 strain, *P2 Streptomyces* Merv 7409 strain

against Gram-positive bacteria, yeasts, and filamentous fungi. On the other hand, the three fusants F5, F8, and F10 were similar to the second parent (P2, Merv 7409); they produced antifungal metabolites (yeasts and filamentous fungi). On the other hand, the fusants F7 and F11 exhibited antibacterial activity against Gram-positive bacteria as the same of the first parent (P1, Merv 1996). The fusants F1, F4, and F6 showed inhibition activity against Gram-positive bacteria and yeasts, while fusant F3 displayed an activity against Gram-positive bacteria and filamentous fungi.

New recombination of microorganisms via protoplast fusion is considered as one of the recent techniques that plays an important role in industrial strains improvement for the production of much interested bioactive secondary metabolites. This might be helpful for the development of a cure for recent diseases and drug-resistant phenomena as well as in the development of pharmaceutical, agrochemical, and biochemical agents and their lead compounds. Many authors studied the effect of the protoplast fusion technique on antibiotics productivity, and as a tool in gene transfer to construct new recombinants with new metabolites. Vla et al. [35] got about 150–200% improvement in productivity of *S. fradia* (the producer of tylosin) after protoplast fusion. Moreover, Wesseling and Lago [36] obtained new recombinants when they fused protoplast between *S. lactamurans* and *S. griseus*. On the other hand, Khattab and EL-Bondkly [3] used the protoplast fusion protocol to improve the productivity of nystatin and antibacterial antibiotics produced by *S. noursei* NRRL 1714.

Based on the obtained results, fusants F2 and F9 were established as the promising recombinant isolates for the production of antibacterial and antifungal activities; that is, they contain the heredity characteristics of both parents, Merv 1996 and 7409. This was further established by our chemical screening using TLC, confirming the existence of the

three bioactive detectable bands, the one sky blue band of Merv 1996 and the two yellow bands of Merv 7409, respectively, compared with the bioactive isolated compounds (**1–3**; Fig. 3). The panel shown on the left (Fig. 3a) displayed the three compounds as fluorescence bands at 366 nm (during a comparison with their parental strains versus the selected fusant) while the right one (Fig. 3b) after spraying with sulfuric acid and heating. Therefore, we have selected F2 for further studies. The recombinant isolate was subjected to upscaling fermentation, followed by isolation, purification, and identification of the bioactive compounds.

Fermentation and Working Up

During our fermentation time course, the production of the 7-methyl coumarin (**1**) in the culture broth of *Streptomyces* Merv 1996 strain (P1) started at day 2 and reached its maximum at day 5. On the other hand, the formation of the flavonoid compounds, rhamnazin (**2**) and cirsimaritins (**3**), in the fermentation broth of *Streptomyces* Merv 7409 strain (P2) began at day 2 and reached maximum at day 7. However, the production of the three bioactive compounds (**1–3**) by the fusant F2 initiated at day 3 and reached their maximum at day 7.

7-Methylcoumarin

Compound **1** is made up of bright colorless crystals, with UV-blue fluorescence and absorbing properties. It gives no coloration by spraying with anisaldehyde/sulfuric acid or sulfuric acid reagent. It is completely soluble in chloroform, acetone, methanol, and diethyl

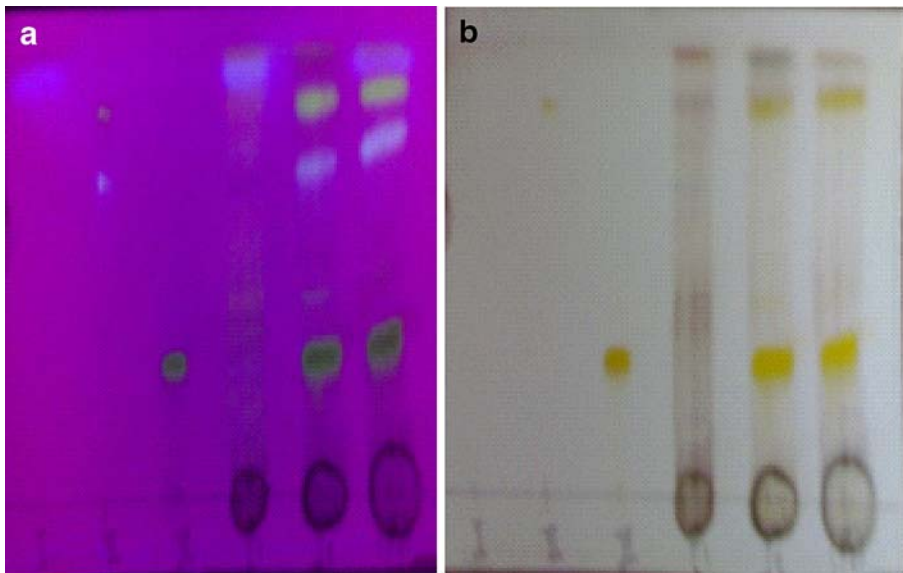


Fig. 3 TLC of the bioactive compounds (**1–3**) compared with that of their producing strains (from left: **1**, **2**, **3**, P1, P2, and F2). **a** The compounds are displayed as fluorescence bands at 366 nm. **b** The compounds are displayed after spraying with sulfuric acid and heating. **1** 7-Methylcoumarin, **2** rhamnazin, **3** cirsimaritin, P1 the extract of *Streptomyces* Merv 1996 strain, P2 the extract of *Streptomyces* Merv 7409 strain, F2 the extract of fusant F2

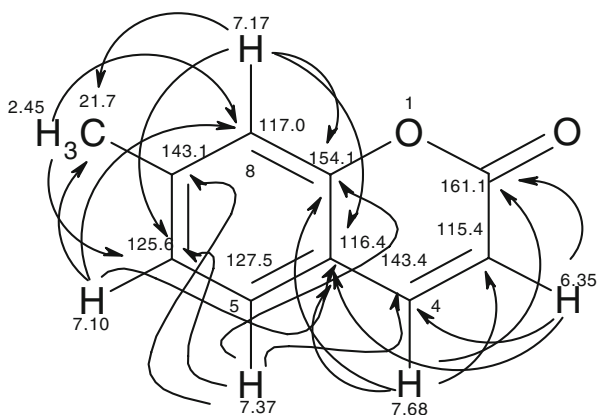
ether. Molecular mass of compound **1** was determined as 160 Da, and EI-HRMS revealed its molecular formula as $C_{10}H_8O_2$.

1H NMR spectra of compound **1** exhibited five aromatic proton signals; two of them were corresponding to *o*-coupled protons (δ 7.68 and 6.35), mostly of an olefinic cis-configuration ($J \sim 9.5$ Hz), and might be conjugated with a carbonyl group. The remaining three protons were of a 1,2,4-trisubstituted aromatic system, two of which were *o*-coupled and appeared at δ 7.37 (d, $J \sim 7.8$ Hz) and 7.10 (dd, $J \sim 7.8$ and 1.1 Hz), while the third one was *m*-coupled and appeared at δ 7.17 (d, $J \sim 1.1$ Hz). In the aliphatic region, one singlet aromatic bounded methyl group was at δ 2.45. The compound bears seven double equivalents; four of them are belonging to an aromatic ring, one of olefinic double, while the residual two are attributed to a second ring closure of lactone.

From the ^{13}C NMR spectra supported by heteronuclear multiple quantum coherence experiment, compound **1** displayed ten carbon signals; of them, two quaternary sp^2 carbons at δ 161.1 and δ 154.1 corresponding to sp^2 -oxy carbons beside to five- sp^2 methine carbons. Additionally, the methyl carbon at δ 21.7 was confirmed as an aromatic bounded group. From the revealed spectral data, the compound could exhibit a coumarin or isocoumarin system. This is because of the small difference in δ_C (161–163) of the carbonyls in both classes (~ 2 ppm) [37], and hence a massive overlap is carried out mainly between both moieties [37]. Moreover, the location of the methyl group, either at 6- or 7-position, needs to be determined.

Therefore, compound **1** was applied to interpretation by the heteronuclear multiple bond correlation experiment (Figs. 3 and 4). The methine proton H-4 at δ 7.68 displayed three significant correlations (3J), at the carbonyl (δ 161.1), the oxy-carbon (δ 154.1), and CH-5 (δ 127.5). Additionally, the low- δ position of the methine carbon (CH-4) pointed to its location in the β -position to the carbonyl (δ 161.1). Moreover, the methine proton H-8 (δ 7.17) displayed no cross-section at the carbonyl (δ 161.1), confirming their nonpreposition, excluding the isocoumarin categorization. On other hand, the methine proton H-3 (6.35) displayed three cross-sections, at the carbonyl (2J), the β -methine carbon (CH-4, 2J), and C-4a (116.4, 3J). The aromatic bounded methyl group ($\delta_H \sim 2.45$) displayed three correlations, one as 2J at C-7 (δ 143.1) and two 3J at both CH-6 ($\delta_C \sim 125.6$) and CH-8 ($\delta_C \sim 117.0$). Both methine carbons, C-5 and C-8, were deduced because of their exhibited 3J correlations at C-8a (154.1) and C-4a (116.4), respectively. Based on the

Fig. 4 Heteronuclear multiple bond correlation connectivities of 7-methylcoumarin (compound **1**)



detailed discussion of the spectral data, compound **1** was confirmed as 7-methylcoumarin, which is known from plants [38, 39] and synthetic routes [40–42].

Rhamnazin; Quercetin-7,3'-Dimethylether

Compound **2** is a yellow solid, with UV-absorbing behavior. It exhibited an intensive yellow coloration with aluminum chloride as well as ammonia vapor. This is an indicative of compound **2** is a flavonoid with free 5- and 4'-hydroxyl groups. This nature was further confirmed from UV spectral data [43], at where the spectrum in a basic methanol (MeOH/NaOMe) showed a bathochromic shift in band I with increasing intensity. The hypsochromic shift for band II in sodium acetate suggested a substitution of the 7-hydroxy group.

The molecular mass of compound **2** was determined by EI-MS as 330 Da, with a base peak character. It showed additionally two fragment ions at m/z 315 and 300 because of successive expulsion of one and two methyl groups, respectively, from the parent compound.

The ^1H NMR spectrum of compound **2** showed a broad signal at δ 12.83 corresponding to the 5-hydroxyl group. The aromatic protons of the B ring appeared as two doublet signals; the first was attributed to *o*-coupled protons at δ 8.14 and 7.12 ($J=8.0$ Hz) of H-6' and H-5', respectively, while the second one was a *m*-coupled proton at δ 8.17 of H-2'. Two singlets at δ 6.97 and 6.57 were assigned to H-6 and H-8, respectively, while the singlet of H-3 (mainly appeared at δ 6.80) was not displayed, pointing to its blocking by a hydroxyl group. The spectra displayed additionally two aromatic bounded methyl ethers at δ 3.72 and 3.69. These data pointed to rhamnazin; quercetin-7,3'-dimethylether (**2**) [25, 44].

Cirsimaritin; 6-Methoxyapigenin 7-Methylether

Compound **3**, obtained as a yellow solid, was also shown to exhibit the same UV characters with aluminum chloride and ammonia vapor as compound **2**, indicating its flavonoid nature, with free 5- and 4'-hydroxyl groups. This character was further confirmed from UV spectral data.

Table 2 Minimum inhibition concentration of compounds 1–3.

Test microorganism	MIC ($\mu\text{g/ml}$)		
	1	2	3
<i>E. coli</i> (ATCC 10536)	0	0	0
<i>P. aeruginosa</i> (ATCC 10145)	0	0	0
<i>B. subtilis</i> (ATCC 6051)	3.6	0	0
<i>S. aureus</i> (ATCC 6538)	0.8	0	0
<i>M. luteus</i> (ATCC 9341)	2.0	0	0
<i>R. minuta</i>	0	0	1.0
<i>P. angusta</i>	0	0	1.0
<i>C. albicans</i>	0	0	1.0
<i>C. neoformans</i>	0	0	1.0
<i>A. niger</i>	0	1.0	0
<i>B. fabae</i>	0	2.5	0

MIC Minimum inhibition concentration, 1 7-methylcoumarin, 2 rhamnazin, 3 cirsimaritin

EI-MS spectrum indicated a $[M]^+$ at m/z 314 as a base peak and two fragment ions at m/z 299 (75%) $[M-CH_3]^+$ and 271 ($[M-CO+CH_3]^+$, 28%). The 1H NMR spectrum of compound **3** showed a broad signal at δ 12.85 corresponding to a 5-hydroxyl group. The aromatic protons of the B ring appeared as a two doublet signals at δ 7.95 and 6.95 with $J \sim 8.0$ Hz due to ortho-coupled protons assigned to H-2',6' $\alpha\delta$ H-3',5', respectively. Two singlets at δ 6.92 and 6.80 were assigned to H-8 and H-3. In addition, the spectrum showed two singlets at δ 3.95 and 3.85 for two methoxy groups. These data pointed to compound **3** as cirsimaritin; 6-methoxyapigenin 7-methylether [45].

Although compounds **1–3** are known as plant metabolites [38, 39, 44, 45] or as synthetic products [37, 39], this is the first reported occurrence as microbial products.

Minimum Inhibition Concentration

7-Methylcoumarin (**1**) is equipotent in inhibiting the growth of *S. aureus* and the growth of Gram-positive bacteria within a concentration of 0.8–3.6 μ g/ml. However, it displayed no effects against Gram-negative bacteria and fungi. Quercetin-7,3'-dimethylether (**2**) showed powerful activities against filamentous fungi *A. niger* and *B. fabae* with a concentration between 1.0 and 2.5 μ g/ml, respectively. In spite of the nonantibacterial activity of cirsimaritin (**3**), it displayed a potent antifungal activity in vitro against *Rhodotorula minuta*, *P. angusta*, *C. albicans*, and *C. neoformans* with a MIC of ~ 1 μ g/ml with no activity against filamentous fungi (Table 2).

References

1. Elyakov, G. B., Kuznetsova, T. A., Stonik, V. A., & Mikhailov, V. V. (1994). *Pure and Applied Chemistry*, 66(4), 811.
2. Arakawa, K., Sugino, F., Kodama, K., Ishii, T., & Kinashi, H. (2005). *Chemistry & Biology*, 12, 249.
3. Khattab, A. A., & EL-Bondkly, A. M. (2006). *Arab Journal of Biotechnology*, 9, 95.
4. Ikeda, H., Inoue, M., & Omura, S. (1983). *Journal of Antibiotics*, 36, 283.
5. Lukyanchuk, V. V., & Matselyukh, A. (1996). *Mikrobiologicheskii Zhurnal*, 58, 382.
6. EL-Bondkly, A. M., & Khattab, A. A. (2004). *Egyptian Journal of Genetics and Cytology*, 33, 217.
7. Kuhnau, J. (1976). *World Review of Nutrition and Dietetics*, 24, 117.
8. Hetreg, M., Hollman, P., Katan, M., & Kromhout, D. (1993). *Nutrition and Cancer*, 20, 21.
9. Croft, K. D. (1998). *Annals of the New York Academy of Sciences*, 854, 435.
10. Di Carlo, G., Mascolo, N., Izzo, A. A., & Capasso, F. (1999). *Life Sciences*, 65, 337.
11. Renaud, S., & de Lorgeril, M. (1992). *Lancet*, 339, 1523.
12. Fuhrman, B., Buch, S., Vaya, J., Belinky, P. A., Coleman, R., Hayek, T., et al. (1997). *American Journal of Clinical Nutrition*, 66, 267.
13. Vaya, J., Belinky, P. A., & Aviram, M. (1997). *Free Radical Biology & Medicine*, 23, 302.
14. Ruiz-larrea, M. B., Mohan, A. R., Paganga, G., Miller, N. J., Bolwell, G. P., & Rice-Evans, C. A. (1997). *Free Radical Research*, 26, 63.
15. Van het Hof, K. H., de Boer, H. S., Wiseman, S. A., Lien, N., Westrate, J. A., & Tijburg, L. B. (1997). *American Journal of Clinical Nutrition*, 66, 1125.
16. Aviram, M., Dornfeld, L., Rosenblat, M., Volkova, N., & Kaplan, M. (2000). *American Journal of Clinical Nutrition*, 71, 1062.
17. Vistioli, F., & Galli, C. (1995). *Nutrition and Metabolism Cardiovascular Diseases*, 5, 306.
18. Fuhrman, B., Rosenblat, M., Coleman, R., Hayek, T., & Aviram, M. (2000). *Journal of Nutrition*, 130, 1124.
19. Phelps, S., & Harris, W. S. (1993). *Lipids*, 28, 475.
20. Sahelian, R. *Coumarin*. <http://www.raysahelian.com/coumarin.html>.
21. Ojala, T. (2001). Academic Dissertation, Division of Pharmacognosy, Department of Pharmacy, Faculty of Science, University of Helsinki.
22. Okamoto, T., Kobayashi, T., & Yoshida, S. (2005). *Current Medical Chemistry: Anticancer Agents*, 5, 47.

23. Weinmann, I. (1997). History of the development and applications of coumarin and coumarin-related compounds. In R. O'Kennedy, & R. D. Thorne (Eds.) *Coumarins: Biology, applications and mode of action* (pp. 1–22). Chichester: Wiley.
24. Matern, U., Lüer, P., & Kreuzsch, D. (1999). Biosynthesis of coumarins. In D. Barton, K. Nakanishi, O. Meth-Cohn, & U. Sankawa (Eds.) *Comprehensive natural products chemistry* (p. 623). Oxford: Elsevier.
25. *Dictionary of Natural Products on CD-ROM* (2005). Chapman & Hall Chemical Database.
26. Laatsch, H. (2004). *AntiBase, a data base for rapid structural determination of microbial natural products, and annual updates*. Weinheim, Germany: Chemical Concepts <http://www.user.gwdg.de/~ucoc/laatsch/AntiBase.htm>.
27. EL-Gendy, M. M. A. (2006). Ph.D. Dissertation, Faculty of Agriculture, Cairo University, Egypt.
28. Büchanan, R. E., & Gibbons, N. E. (1974). *Bergey's manual of determinative bacteriology* (8th ed.). Baltimore, MD: Williams and Wilkins.
29. Streptomyces and related genera (1989). In: *Bergey's manual of systematic bacteriology*, vol. 4, section 29. Baltimore, MD: Williams and Wilkins.
30. Yamaguchi, T. (1965). *Journal of Bacteriology*, 89, 444.
31. Yan, L. P., Hong, K., Hu, S., & Liu, L. H. (2005). *Wei Sheng Wu Xue Bae*, 45, 185.
32. Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., et al. (1985). *Genetic manipulation of Streptomyces. A laboratory manual*. Norwich: John Innes Foundation.
33. Illing, G. T., Normansell, I. D., & Peberdy, F. (1989). *Journal of General Microbiology*, 135, 2289.
34. Curragh, H. J., & Collins, M. A. (1992). *Journal of Applied Bacteriology*, 73, 31.
35. Vla, L. H., Stryzhkora, H., & Mateliukh, P. (1997). *Antibiotic Modbioteckhnologia*, 33, 883.
36. Wesseling, A. C., & Lago, B. D. (1981). *Developments in Industrial Microbiology*, 22, 641.
37. ACD NMR simulation programs Vers. 3.00 (1998). Toronto: Advanced Chemistry Development.
38. Gonzalez, G. A., & Estevez, R. R. (1971). *Farmacia Nueva*, 36, 995, 1003 CA (1972): 76,144738.
39. Patel, D. J., & Bafna, S. L. (1966). *Nature*, 211, 963 CA (1966). 65, 103178.
40. Yoshino, T., Kijima, I., Sugiura, M., & Furuya, R. (1963). *Yuki Gosei Kagaku Kyokaiishi*, 21, 373 CA (1963), 59, 441564.
41. Kitagawa, H., Iwaki, R., Yanagi, B., & Sato, T. (1956). *Yakugaku Zasshi*, 76, 186 CA (1956), 50, 74952.
42. Shalaby, N. M. M., & El-Gamal, M. H. A. (2001). *Egyptian Journal of Chemistry*, 44, 201.
43. Mabry, T. J., Markham, K. R., & Thomas, M. B. (1970). *The systematic identification of flavonoids*. New York: Springer.
44. Wollenweber, E., & Mann, K. (1984). *Zeitschrift für Naturforschung*, C39, 303.
45. Hasrat, J. A., Pieters, L., Claeys, M., Vlietinck, A., De Backer, J., & Vauquelin, G. (1997). *Journal of Natural Products*, 60, 638.